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Interplay Between LRRK2 GTPase and Kinase Domains

Bachelor's thesis

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Abstract

Mutations in leucine-rich repeat-kinase 2 (LRRK2) are a common cause of both familial and sporadic forms of Parkinson's disease (PD). LRRK2 is a large, irregular protein featuring both a kinase and a Ras of complex (Roc) GTPase domain. While these domains can interact in separate proteins, the interplay between the Roc and kinase domain of LRRK2 is still under debate. In this thesis, I delved into the structure and function of both domains, and aimed to find out if there is interplay between them. I found several lines of evidence suggesting interplay between both domains, including effects of GTP binding on LRRK2 dimerisation and kinase activity, phosphorylation of the Roc domain by the kinase domain, and effects of GTP hydrolysis on kinase activity. A deeper understanding of the complex regulatory interactions of LRRK2 Roc and kinase domains will contribute to our understanding of LRRK2 regulation, and will therefore be invaluable for selective pharmaceutical intervention of PD.

1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder affecting 1-2% of people older than 65 years. In recent decades, mutations in the leucine-rich repeat-kinase 2 (LRRK2) gene have been linked to both familial and sporadic forms of PD (Healy et al., 2008).

LRRK2 is an unusually large protein of 286 kDa with a complex domain architecture including four domains involved in protein-protein interactions (PPIs) and two catalytic domains conferring distinct enzymatic activities. The PPI domains consist of an N-terminal ankyrin repeat domain, followed by an armadillo repeat, and a leucine-rich repeat domain, while the final PPI domain, WD40, is located at the extreme C-terminus of LRRK2 (Fig. 1). The C-terminal catalytic domains consist of a Ras of complex (Roc) Ras-like GTPase domain followed by a C-terminal of Roc (COR) domain. This specific domain architecture in which Roc is followed by a COR domain is highly conserved and places LRRK2 in the Roco superfamily of proteins (Bosgraaf & Van Haastert, 2003). Following the RocCOR tandem domain at the C-terminal end of LRRK2 is a mitogen-activated kinase kinase kinase (MAPKKK)-like kinase domain (Gloeckner et al., 2009).

At least seven missense mutations segregating with PD have been identified, all located in LRRK2 catalytic domains: N1437H and R1441C/G/H in the Roc domain, Y1699C in the COR domain, and G2019S and I2020T both in the kinase domain (West et al., 2005). The most frequent PD mutation, G2019S, enhances kinase activity (Steger et al., 2016). Therefore, the kinase domain has been researched intensively, and most pharmacological interventions are aimed at the kinase activity. However, of all LRRK2 mutations segregating with PD, only the G2019S mutation has been shown to significantly enhance kinase activity in vitro, while all other PD-related mutations only seem to increase kinase activity in a cellular context (Steger et al., 2016). While more than half of the currently identified LRRK2 mutants are located in the RocCOR tandem domain, detailed information on the functions and regulation of LRRK2 GTPase activity is currently lacking.

One major hurdle in LRRK2 research has been the lack of high-resolution crystal structures, as the large and flexible nature of LRRK2 has caused difficulties in obtaining large amounts of pure, stable protein. Recently, Guaitoli et al. (2016) generated a 3D model of dimeric, full-length LRRK2 by combining homology models from a *Chlorobium tepidum* LRRK2 analogue, CtRoco, with multiple experimental constrains provided by a range of biochemical and biophysical methods. High-resolution structural data of the C-terminal domains of human LRRK2 has also recently become available, giving important insight in the structure of both catalytic domains as well as the C-terminal WD40 domain (Deniston et al., 2020).

MAPKKK kinases and Ras GTPases are frequently part of the same regulatory cycle, exemplified by the regulation of RAF protein kinase by Ras-GTPases (Lavoie & Therrien, 2015). As LRRK2 contains both these domains, it is not unlikely that there exists an intramolecular regulatory

interaction between them. However, the interplay between LRRK2 Roc and kinase domains remains a subject of debate in literature. In this thesis, I aim to find out if there is any interplay between the kinase and GTPase domains of LRRK2. For this, I delve into the structure and function of both domains, and try to find evidence of interactions between them by looking at LRRK2 substrates, functional LRRK2 mutations, and the effect of GTP binding and hydrolysis on LRRK2 kinase activity. Lastly, I summarise different models of LRRK2 activation, and propose an integrated model for LRRK2 activation.

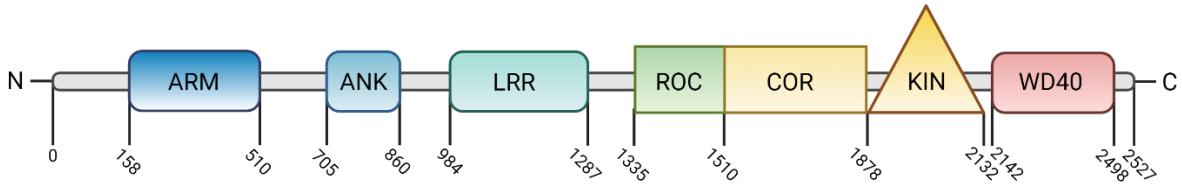

Figure 1: Domain architecture of LRRK2. The three N-terminal PPI domains consisting of armadillo repeats (ARM), ankyrin repeats (ANK) and leucine-rich repeats (LRR) are followed by the Ras-like GTPase Ras of complex (Roc) C-terminal of Ras (COR) tandem domain, MAPKKK-like kinase domain, and at the extreme C-terminal the fourth PPI domain, WD40.

2. Structure and function of LRRK2 kinase domain

LRRK2 kinase activity has been in the spotlight since the discovery that the most frequent PD-pathogenic LRRK2 mutation, G2019S, causes an increase in kinase activity (Steger et al., 2016). Based on sequence similarity, LRRK2 kinase belongs to the MAPKKK subclass of RAF kinases that frequently play a role in cell growth, cell differentiation, apoptosis, and inflammation (Alberts et al., 2015; Lavoie & Therrien, 2015). Indeed, Gloeckner et al. (2009) showed that LRRK2 phosphorylates downstream mitogen-activated kinase kinases, a well characterised substrate of MAPKKKs involved in stress-responses, in vitro. Correspondingly, mutations elevating LRRK2 kinase activity have been linked not only to PD, but also to several bacterial infections, leprosy, and inflammatory bowel disease, indicating a role of LRRK2 kinase activity in inflammatory responses (Wallings & Tansey, 2019).

LRRK2 substrates have been hard to confirm in vivo. Recently, Steger et al. (2017) discovered a subset of Rab GTPases as bona fide substrates of LRRK2 kinase activity. Rab3A/B/C/D, Rab5A/B/C, Rab8A/B, Rab10, Rab12, Rab29, Rab35, and Rab43, which play a role in intracellular vesicular trafficking, are phosphorylated by LRRK2 in vivo (Steger et al., 2017). These Rab GTPases fulfil functions in endosomal trafficking (Rab5), T-cell-migration (Rab5), hormone secretion (Rab8), regulation of cioliogenesis (Rab8), centrosomal cohesion (Rab8), cilia formation (Rab10), sonic hedgehog signalling (Rab10), phosphorylation-dependent binding to Rab interaction lysosomal protein like 1 and 2 (RILPL1/2, Rab12), regulation of LRRK2 (Rab8/10/29), and α-synuclein propagation (Rab35), illustrating the wide variety of cellular functions LRRK2 regulates (Waschbüsch & Khan, 2020).

Besides phosphorylating proteins in *trans*, LRRK2 is also capable of phosphorylation in *cis*. The N-terminal autophosphorylation cluster consisting of S910, S935, S955 and S973 plays a role in phosphorylation-dependent binding of scaffold protein 14-3-3 to LRRK2 (Doggett et al., 2011; Dzamko et al., 2010). In vitro studies could not confirm that LRRK2 phosphorylates these residues, indicating that these residues might be phosphorylated by an external protein kinase or that external factors not present in purified LRRK2 are required for autophosphorylation of these sites (Dzamko et al., 2010; Purlyte et al., 2018). Several LRRK2 residues have been confirmed to be autophosphorylated both in vitro and in vivo. Autophosphorylation of a threonine cluster in the Roc domain is characterised by increased GTP hydrolysis and promoted dimerisation, suggesting an autoregulatory role between the kinase and GTPase activity of LRRK2 (Liu et al., 2015). Sheng et al. (2012) convincingly showed that

S1292, located between the LRR and Roc domain of LRRK2, is autophosphorylated in vivo and serves as a direct readout of kinase activity. Though the exact function of the autophosphorylation site is unclear, it has been shown that several PD mutations located in both the kinase and GTPase domains of LRRK2 cause increased phosphorylation of S1292, and that altered S1292 autophosphorylation may play a role in regulating kinase activity (Sheng et al., 2012). In recent structures of the LRRK2, the S1292 residue seems to be located close to the kinase active site, supporting a role for S1292 phosphorylation in regulating kinase activity (Deniston et al., 2020).

Due to the large and flexible nature of LRRK2, structural data of LRRK2 has been hard to come by. High-resolution structures of LRRK2 have been confined to structures of LRRK2 homologs, truncated human LRRK2, or inhibitor-bound LRRK2. Recently, a relatively high-resolution structure (3.5 Å) was obtained of the open, inactive conformation of the monomeric C-terminal domains of LRRK2, Roc, COR, kinase and WD40 referred to as RCKW (Deniston et al., 2020, reviewed in Taylor et al., 2020). Protein kinases function in signal transduction pathways, regulating their substrates through phosphorylation. Though the occurrence of both a kinase and a GTPase domain in a single protein is rare, the architecture of the LRRK2 kinase domain is conserved, and places it in the MAPKKK superfamily of protein kinases (Gloeckner et al., 2009). The architecture of LRRK2 kinase domain is highlighted in Figure 2A. The LRRK2 kinase domain consists of two subdomains, the N- and C-lobe. The N-lobe consists of five β-sheets, β1-β5, spanning the entire lobe, with the αC-helix between β4 and β5. The C-lobe consists of six alpha helices termed αA-αF, with an activation segment between the αB and αC helices consisting of an activation loop referred to as the G-loop and a P + 1 loop linked to the αF helix that spans the C-lobe (Taylor et al., 2020). Flanking the N-terminal of the activation segment is the DYG motif (in most kinases DFG), which is important for binding Mg^{2+} and ATP (Taylor et al., 2020). The most prevalent LRRK2 mutant G2019S lies in this motif, which is believed to lead to a local alteration in the amino acid backbone that is likely responsible for the permanent “on” state of the activation segment (Gloeckner et al., 2009). Besides the activation segment responsible for nucleotide binding, protein kinases also contain a regulatory and catalytic spine, the R-spine and C-spine, respectively. The R-spine consists of four residues that are disassembled in an inactive state, and assembled in an active conformation, thereby connecting the N- and the C-lobes. The C-spine is localised in the N-lobe and consists of two residues that together form a hydrophobic cap for the buried adenine ring of ATP (Taylor et al., 2020). For most protein kinases, an inactive conformation is characterised by a disassembled R-spine, an ordered activation segment, and the DFG motif in an “out” conformation. An active conformation is represented by an assembled R-spine, a closed conformation around the bound ATP, a disordered activation segment, and a DFG “in” conformation (Taylor et al., 2020).

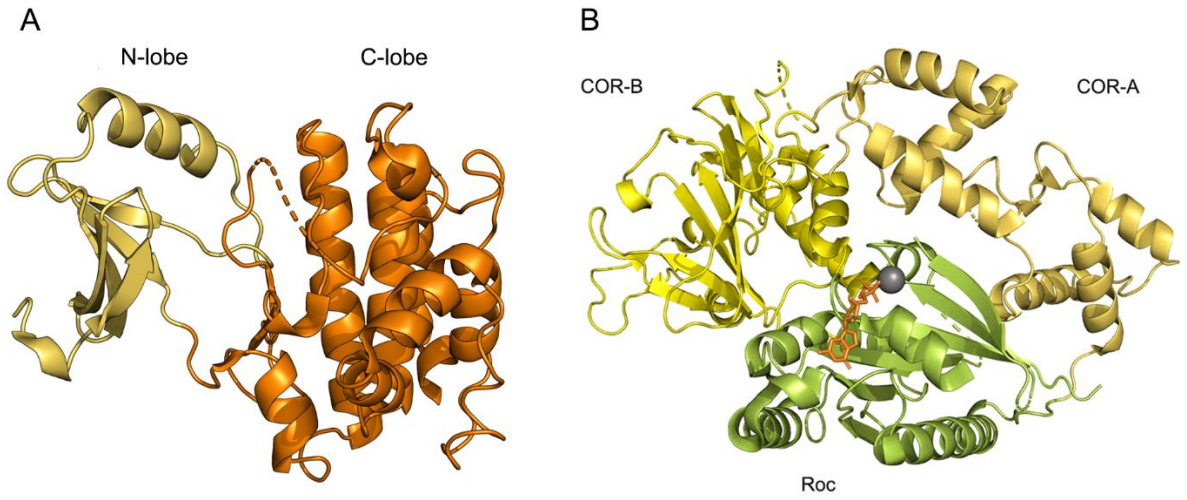

Figure 2: Structure of the catalytic domains of human LRRK2 with the kinase domain in its apo state and the RocCOR tandem domain bound by GDP and Mg^{2+} (PDB: 3VNO). A) The kinase domain of LRRK2 consist of an N- and C-lobe. The N-lobe (beige) consists of five β-sheets, β1-β5, spanning the entire lobe, with the αC-helix between β4 and β5. The C-lobe (orange) consists of six α-helices termed αA-αF, with an activation segment between the αB and αC helices consisting of an activation loop referred to as the G-loop and a P + 1 loop linked to the αF helix that spans the C-lobe. B) The Roc domain of LRRK2 consists of six β-strands surrounded by five α-helices connected by hydrophilic loops. Immediately following the Roc domain is the COR domain, consisting of two subdomains. COR-A (beige) consists of six α-helices, while the adjacent COR-B (yellow) consists of six β-sheets and four α-helices.

3. Structure and function of LRRK2 GTPase domain

The GTPase domain of LRRK2 has received less attention than the kinase domain. LRRK2 GTPase consists of a Ras-like GTPase domain, Roc, directly followed by the C-terminal of Roc or COR domain, as is conserved throughout all Roco proteins (Bosgraaf & Van Haastert, 2003). Four Roco proteins have been characterised in vertebrates, their human homologs being death-associated protein kinase 1 (DAPK1), malignant fibrous histiocytoma amplified sequence 1 (MFHS1), leucine-rich repeat kinase 1 (LRRK1), and LRRK2 (Wauters et al., 2019). Especially DAPK1 nicely illustrates the interplay between this protein's kinase and GTPase activity. Binding of GTP in the DAPK1 GTPase domain causes a conformational change in its kinase domain, leading to autophosphorylation of the calmodulin-regulatory domain, and a decrease in kinase activity. Upon GTP hydrolysis, DAPK1 kinase activity is reactivated (Wauters et al., 2019). Roco proteins fulfil a range of physiological functions including regulation of cell death and autophagy (DAPK1), B-cell receptor-mediated B-cell proliferation and survival and endosomal sorting (LRRK1), and mitochondrial fission and fusion dynamics, regulation of neuronal development, regulation of intracellular vesicular trafficking, and regulation of the production of inflammatory cytokines (LRRK2, Wauters et al., 2019).

Classical GTPases act as molecular switches that bind GTP in their active state and GDP in their inactive state. For classical GTPases, GTP binding and hydrolysis is commonly regulated by GTP-exchange factors (GEFs), which mediate the exchange of GDP by GTP to activate the GTPase, and GTPase-activating proteins (GAPs) stimulating GTP hydrolysis, thereby inactivating the GTPase (Alberts et al., 2015). Some GTPases belong to the class guanine nucleotide-binding (G) protein activated by nucleotide-dependent dimerisation (GAD). GADs function independent of GEFs and GAPs, and are instead activated by guanine nucleotide-dependent dimerisation (Gasper et al., 2009). Though LRRK2 shows low nucleotide affinity and increased GTPase activity upon dimerisation characteristic of GADs, LRRK2 GTPase also shows interaction with GEFs and GAPs (Nguyen & Moore, 2017). In vitro studies show that the GEF AhrGEF7 binds LRRK2 and regulates its GTPase activity, and two GAPs, ArfGAP1 and RGS2, have been shown to increase in vitro GTP hydrolysis (Nguyen & Moore, 2017).

Therefore, it is currently unclear if LRRK2 GTPase activity is regulated similar to classical GTPases, GADs, or if the LRRK2 G-protein cycle follows a different mechanism.

Another functional property of the RocCOR tandem domain of LRRK2 associated with its GAD functionality is dimerisation. The COR domain of LRRK2 is considered to be its main dimerisation device, though there is also evidence that the WD40 domain is required for dimerisation and that the N-terminal PPI domains of LRRK2 mediate dimerisation (reviewed in Civiero et al., 2017). In contrast to normal GADs, in which dimerisation is associated with GTP binding and increased GTP hydrolysis, a bacterial homologue of LRRK2 from *C. tepidum*, CtRoco, was shown to undergo a G-protein cycle in which binding of GTP induces monomerisation, while GTP hydrolysis and GDP release is coupled to dimerisation (Deyaert et al., 2017). However, the dimerisation interface of the dimer modelled based on *C.tepidum* LRR-Roc-COR contains an interface reliant on the Roc domain, while a human LRRK2 RCKW structure shows dimerisation exclusively mediated by the COR domain (Deniston et al., 2020). As a result, GTP hydrolysis cycles might have different effects on dimerisation in human LRRK2. Moreover, recent data by Schmidt et al. (2021) shows that the N-terminal PPI domains not present in the RCKW version of human LRRK2 nor CtRoco form a "lid" for the kinase domain, keeping it inactive. Association of Rab proteins with N-terminal domains is said to release the "lid", thereby initiating LRRK2 kinase activation (Purlyte et al., 2018; Schmidt et al., 2021). Though it is clear that LRRK2 dimerisation contributes to GTPase and kinase activity, the exact mechanism by which this is achieved remains to be elucidated.

The Roc domain of LRRK2 harbouring its GTPase activity follows the general architecture common to all small GTPases, highlighted in Figure 2B. The LRRK2 Roc domain consists of a classical G-protein fold: six β-strands surrounded by five α-helices connected by hydrophilic loops. Five G-domain sequences can be found in the Roc domain: the guanine nucleotide phosphate-binding (P)-loop that binds GDP or GTP (G1), the switch I (G2) and II (G3) motifs that change conformation upon GTP binding and regulate GTP hydrolysis, and the guanine specificity determining motifs (G4-G5) (Wauters et al., 2019; Nguyen & Moore, 2017). Immediately following the Roc domain is the COR domain, consisting of two subdomains, COR-A and COR-B. COR-A consists of six α-helices, while the adjacent COR-B consists of six β-sheets and four α-helices. The COR-A and tightly associated Roc domain are folded in close proximity to the C-lobe of the kinase domain, while the αC-helix of the N-lobe of the kinase domain forms an extensive interaction with COR-B (Deniston et al., 2020). This close interaction likely drives cross-talk between the GTPase and kinase domain. Both PD-relevant R1441 and Y1699 lie in the interface of the Roc and COR-B domains, suggesting that these mutations may influence cross-talk between the kinase and RocCOR domains by altering local conformations (Deniston et al., 2020).

4. Interplay between the kinase and GTPase domain

The LRRK2 kinase domain has been a frequent target for pharmaceutical intervention in the treatment of PD. Even though effective LRRK2 kinase inhibitors are already available, they tend to disrupt normal localisation of LRRK2, and do not solely affect its kinase activity (Dzamko et al., 2010). A deeper understanding of the functional impact of PD-associated LRRK2 mutations, the regulation of upstream and downstream effector proteins of LRRK2, and the interdependence between both catalytic domains of LRRK2 could provide valuable information in treating PD. In the next paragraphs, I aim to illustrate the interplay between LRRK2 kinase and GTPase functions by investigating the role of GTP binding and hydrolysis on kinase activity, the regulation of LRRK2 effector proteins, and the effect of different mutations on both domains.

4.1 Rab GTPases illustrate the interplay between LRRK2 kinase and GTPase activity

One thing that has hampered research on the catalytic activity of LRRK2 is the lack of reliable LRRK2 substrates. Several substrates have been shown to interact with LRRK2 in vitro, like moesin and Rps15, but evidence of their interaction in vivo is currently lacking (Steger et al., 2016). Using highly stringent phosphoproteomics methods combined with genetic, biochemical, and pharmacological approaches, Steger et al. (2016) found that a subset of Rab GTPases is phosphorylated by LRRK2 in vivo. As mentioned earlier, the same group later confirmed this phosphorylation for Rab3A/B/C, Rab8A/B, Rab10, Rab12, Rab35 and Rab43 (Steger et al., 2017).

Rab GTPases consist of a large superfamily of over 60 members playing a major role in the specificity of vesicular transport (Alberts et al., 2015). Rab proteins cycle between a GDP-bound inactive state, in which they are stabilised in the cytosol by GDP-dissociation inhibitors (GDIs), and a GTP-bound active state, in which they are embedded in a membrane (Alberts et al., 2015).

Rab GTPases are phosphorylated by LRRK2 in conserved residues in their switch II motif, which regulates GTP hydrolysis as well as interactions with Rab effector proteins (Steger et al., 2016). For example, Rab phosphorylation in the switch II motif by LRRK2 has been found to decrease Rab affinity to Rab GDIs, GEFs and GAPs, while it increases affinity for proteins such as RILPL1/2 implicated in ciliogenesis (Steger et al., 2017; Waschbüsch & Khan, 2020). This way, kinase activity of LRRK2 can regulate numerous endosomal vesicular trafficking pathways by phosphorylation of Rab GTPases. Interestingly, mutations found in the GTPase domain of LRRK2 increase the phosphorylation of Rab GTPases, suggesting a role of GTPase activity in the regulation of LRRK2-mediated Rab phosphorylation (Steger et al., 2017). This is in contrast with the finding that mutations in the RocCOR tandem domain show no increase in kinase activity in vitro, and implies that increased phosphorylation of LRRK2 substrates in vivo caused by GTPase mutations is due to a different mechanism (Greggio & Cookson, 2009; Taymans et al., 2011). As Rab GTPases are phosphorylated by LRRK2 and both Rab GTPases and LRRK2 cycle between inactive cytosolic and active membrane-associated phases, it is likely that the increased kinase activity of LRRK2 GTPase mutants is due to increased co-localisation of both proteins (Steger et al., 2017).

Though most Rab GTPases phosphorylated by LRRK2 only seem to be part of downstream signalling pathways of LRRK2, Rab29 also functions as an upstream effector of LRRK2 (Purlyte et al., 2018; Waschbüsch & Khan, 2020). Rab29 plays a role in intracellular vesicular trafficking at the trans-Golgi network (TGN) and has been shown to activate LRRK2 kinase activity by binding to the ankyrin domain of LRRK2 (Purlyte et al., 2018). Overexpression of Rab29 increases phosphorylation of LRRK2 autophosphorylation site S1292, as well the N-terminal autophosphorylation cluster S910, S935 S955 and S973, and Rab10 in wild-type, RocCOR mutant, and G2019S mutant LRRK2. Rab29 overexpression is also characterised by increased co-localisation of LRRK2 with Rab29 at the TGN, while disruption of Rab29 binding to LRRK2 prevents this co-localisation, as well as the Rab29-mediated increase in kinase activity. Interestingly, GTP binding deficient LRRK2 mutants show phosphorylation in neither *cis* nor *trans*, suggesting that GTP binding is critically required for the Rab29-mediated activation of LRRK2 kinase activity (Purlyte et al., 2018).

Besides being an upstream regulator of LRRK2, Rab29 also forms a substrate for LRRK2 phosphorylation (Steger et al., 2017). Phosphorylation of Rab29 at threonine residues in its switch II motif seems to suppress Rab29-mediated activation of LRRK2 kinase activity, indicating that Rab29 phosphorylation might be part of a negative feedback loop that terminates LRRK2 activity (Purlyte et al., 2018).

We thus arrive at a model in which GTP binding via the Roc domain of LRRK2 allows it to localise to membranes, where LRRK2 is bound and activated by Rab GTPases, stimulating LRRK2-dependent phosphorylation of downstream Rab proteins and LRRK2 autophosphorylation. Phosphorylated downstream Rab proteins have altered affinity towards their effector, leading to

changes in downstream signalling pathways.

4.2 Effect of GTP capacity and hydrolysis on LRRK2 kinase activity

An obvious example of the interplay between LRRK2 GTPase and kinase domains is through the alteration of kinase activity upon binding of guanine nucleotides to the Roc domain. Several studies have probed the contribution of guanine nucleotide binding to kinase activity with different results. Using radiolabelled P^{32}, Taymans et al. (2011) assessed this contribution by measuring autophosphorylation of LRRK2 and phosphorylation of LRRKtide, a well-characterised LRRK2 substrate, for different LRRK2 mutants in the presence or absence of GDP, GTP, and GTP's non-hydrolysable counterparts, GTPγS and GppCp. Their data showed no significant difference in kinase activity between purified LRRK2 treated with GDP, GTP or its non-hydrolysable counterparts, while cell lysates treated with guanine nucleotides prior to LRRK2 purification showed increased kinase activity for GTPγS and GppCp compared to GDP-treated lysates, indicating that additional factors may contribute to GTP-dependent kinase activation in vivo (Taymans et al., 2011).

In a similar study, Biosa et al. (2012) measured the effect of addition of GDP, GTP and their non-hydrolysable counterparts, GDPβS and GppCp, respectively, on LRRKtide phosphorylation in vivo using radioactive kinase assays. They found that GTP and GppCp enhanced, while GDP but not GDPβS reduced phosphorylation of LRRKtide. GTP enhanced LRRK2 kinase activity to a greater extent than GppCp, suggesting that GTP hydrolysis enhances LRRK2 kinase activity even further, contradicting the findings of Taymans et al. (Biosa et al., 2012). However, both studies show that GTP binding deficient mutants (K1347A and T1348N) show no GTPase activity nor kinase activity, indicating that GTP binding is critically required for normal LRRK2 functioning (Biosa et al., 2012; Taymans et al., 2011).

The binding of GTP itself indeed seems to play a role in LRRK2 activation. PD-associated mutations in the Roc and COR domains of LRRK2 cause a marked redistribution of LRRK2 to stable microtubules, thereby inhibiting microtubule-dependent intracellular trafficking (Blanca Ramírez et al., 2017b). The R1398L mutations in the switch II region of the Roc domain shows increased GTP hydrolysis and reduced GTP binding, while the R1398L/T1343V mutation decreases GTP binding, and filament formation of all LRRK2 pathogenic mutants (Blanca Ramírez et al., 2017b). Correspondingly, LRRK2 mutants showing enhanced kinase activity or filament formation show increased GTP binding. Introducing the R1398L/T1343V mutation decreases kinase activity, filament formation, and GTP binding in most of these mutants. Similarly, the observed protective effects of 14-3-3 binding correlates with reduced GTP binding rather than increased phosphorylation of the N-terminal serine cluster (Blanca Ramírez et al., 2017b). Enhanced GTP binding also seems to play a role in Rab29-mediated activation of LRRK2. LRRK2 mutations with enhanced GTP binding are more readily recruited to the TGN and activated more efficiently by Rab29 than wild-type LRRK2 (Purlyte et al., 2018). Together, these data suggest that the extent of GTP binding dictates LRRK2 localisation and activation. It is worth noting, however, that the effects of both R1398L and R1398L/T1343V are not reconciled between different studies. Biosa et al. (2012) reported that both mutants maintain normal GTP binding, and that the R1398L mutation increases GTP hydrolysis, while the R1398L/T1434V mutant impairs GTP hydrolysis and represents a GTP locked version of LRRK2.

4.3 Effect of functional mutations on the interplay between both domains

As mentioned earlier, seven missense mutations of LRRK2 linking to PD have been described and all are located in the catalytic domains of LRRK2: N1437H and R1441C/G/H in the Roc domain, Y1699C in the COR domain, and G2019S and I2012T in the kinase domain. The most common PD-associated LRRK2 mutation, G2019S, increases LRRK2 kinase activity (West et al., 2005). In vitro studies on the effect of the RocCOR mutations have been contradicting each other, with some studies finding no effect on kinase activity, while others report enhanced kinase activity (Guo et al., 2007; Steger et al.,

2016; West et al., 2005). Interestingly, when looking at the effects of these mutations in a cellular environment, researchers found a marked increase in kinase activity in all PD-associated LRRK2 mutations (Steger et al., 2016; Sheng et al., 2012). As explained in the previous section, LRRK2 mutants show increased co-localisation with Rab proteins, and the increased kinase activity may be due to the increased local concentration of LRRK2 with its Rab substrates.

Another phenomenon that complicates these findings is the marked relocalisation that is observed in RocCOR mutants, but not I2020T and G2019S. RocCOR mutants show filamentous localisation in cells, corresponding to stable microtubules (Blanca Ramírez et al., 2017b). Filamentous localisation of LRRK2 is paralleled by reduced binding of 14-3-3 (Blanca Ramírez et al., 2017b). 14-3-3 Binds LRRK2 in a phosphorylation-dependent manner to a serine cluster consisting of S910, S935, S955 and S973, thereby stabilising LRRK2 in the cytosol (Doggett et al., 2011; Dzamko et al., 2010). Correspondingly, mutants that show filamentous localisation also show reduced phosphorylation of this serine cluster and reduced 14-3-3 binding (Blanca Ramírez et al., 2017b; Doggett et al., 2011). Therefore, while the kinase domain mutants might simply show constitutively enhanced kinase activity, the enhanced kinase activity observed in RocCOR domain LRRK2 mutants might be caused by increased co-localisation of LRRK2 with its substrates (Blanca Ramírez et al., 2017a).

As explained earlier, GTP binding itself also seems to influence filament formation and cellular localisation of LRRK2. The R1441C/G and Y1699C mutants that have been suggested to enhance GTP binding also show filamentous phenotypes, while mutations that increase kinase activity but have no effect on GTP binding like G2019S and I2020T show no filament formation (Guo et al., 2007; Purlyte et al., 2018). These results suggest GTP binding and 14-3-3 binding to LRRK2 might both influence the altered localisation observed in mutant LRRK2.

In a study on the effects of the G2019S LRRK2 mutant on the rodent brain, Nguyen et al. (2020) showed that LRRK2 G2019S-induced neurodegeneration is dependent on both kinase and GTPase activity, contradicting earlier beliefs that the G2019S LRRK2 mutant is independent of GTPase activity. By using neuronal-specific synapsin-1 promotors with different LRRK2 mutants in ad5 viral vectors, they delivered LRRK2 to the substantia nigra pars compacta (SNpc) region of the brain, a region degenerated in PD patients (Nguyen et al., 2020). In their study, they showed that viral packages containing G2019S mutant LRRK2 caused robust neurodegeneration in the SNpc, while combining the G2019S mutation with GTP hydrolysis enhanced R1398L, or GTP binding deficient T1348N mutants had a neuroprotective effect. The R1398L/T1343V mutant reduced autophosphorylation levels and filament formation, while it did not have a significant effect on Rab10 phosphorylation.

Together these data show that LRRK2 kinase activity is enhanced in most PD-associated LRRK2 mutations, either by constitutively enhanced kinase activity or increased co-localisation of LRRK2 with its substrates. Mutations in the GTPase domain of LRRK2 cause mislocalisation of LRRK2 to microtubules, frequently paralleled by loss of phosphorylation of the N-terminal serine cluster and loss of 14-3-3 binding. The cross-talk between LRRK2 kinase and GTPase domains is more indirect, as changes in localisation and kinase activity of LRRK2 seem to be mediated by binding of GTP to the Roc domain of LRRK2. Reduced binding and increased hydrolysis of GTP increase the relative amount of GDP-bound, inactive LRRK2, and has the potential to ameliorate PD-associated pathogenesis.

5. Proposed mechanism of LRRK2 activation

As illustrated in previous sections, there exists some contradiction in the exact mechanisms regarding the functioning of LRRK2 kinase and GTPase domains. As such, several models have been proposed to explain the irregular features of LRRK2 regulation and functioning. In this section, I summarise some of these proposed models, and aim to integrate the information to propose a model myself.

5.1 Proposed mechanisms in literature

Guo et al. (2007) proposed a model similar to a classical G-protein cycle (Fig. 3A). In the model, LRRK2 cycles between a GDP-bound inactive state and GTP-bound active state, facilitated by GEFs and GAPs. They proposed that activation of LRRK2 by GTP binding to its Roc domain stimulates LRRK2 kinase activity, thereby stimulating both autophosphorylation and substrate phosphorylation, with downstream effector functions in neuronal cell signalling.

In a model by Dzamko et al. (2010), LRRK2 is proposed to function as an upstream component of a signal transduction pathway that stimulates the activity of a protein kinase or inhibits the activity of a protein phosphatase that (de)phosphorylates S910 and S935 (Fig. 3B). The phosphorylation status of S910 and S935 dictate the binding of 14-3-3 isoforms that stabilise cytoplasmic LRRK2. Treatment of cells with LRRK2 inhibitors leads to the dephosphorylation of these serine residues, preventing 14-3-3 binding and disrupting LRRK2 localisation (Dzamko et al., 2010).

In a literature study on LRRK2 dimerisation, Civiero et al. (2017) proposed a mechanism of LRRK2 activation via dimerisation analogous to Ras/RAF activation. RAF is stabilised in an inactive conformation in the cytosol by phosphorylation-dependent binding of 14-3-3. Upon activation by extracellular mitogens, GTP-bound Ras interacts with RAF, causing dephosphorylation of RAF, 14-3-3 dissociation, and recruitment of RAF to membranes where it dimerises. Civiero et al. proposed that a similar mechanism is possible for LRRK2, in which LRRK2 forms an inactive monomer stabilised in the cytosol by phosphorylation-dependent 14-3-3 binding. Upon stimulation, LRRK2 could become dephosphorylated by protein phosphatases or by inhibition of protein kinases, causing dissociation of 14-3-3 and relocalisation of LRRK2 to specific membranes, where it dimerises, and activates LRRK2 kinase activity. However, as Civiero et al. points out, LRRK2 phosphorylation status does not always parallel 14-3-3 binding.

Steger et al. (2017) proposed a mechanism of the LRRK2-mediated activation of Rab proteins (Fig. 3C). They proposed a model in which inactive, GDP-bound Rab GTPase are bound in the cytosol by GDIs. LRRK2 mediates the dissociation of Rab GTPases from GDIs and aids the insertion of GDP-bound Rab proteins in their specific target membranes by phosphorylation. Membrane-bound Rabs are dephosphorylated, and GEFs facilitate the exchange of guanine nucleotides, thereby activating the now GTP-bound Rab proteins, which interact with effector proteins. Rab-specific GAPs induce GTP hydrolysis, followed by the removal of the Rab proteins from the membrane by GDIs. In their adapted model for mutant LRRK2, they propose that hyperactive LRRK2 leads to reduced affinity of Rab GTPases to GDIs (Fig. 3D). As a result, the Rab GTPases become increasingly membrane-bound and active, leading to pathogenic phenotypes.

In a similar model, Purlyte et al. (2018) proposed a mechanism for the recruitment of LRRK2 to the TGN and its associated Rab29-mediated kinase activation (Fig. 3E). Their model suggests that GTP binding to the Roc domain of LRRK2 promotes Rab29-mediated kinase activation. LRRK2 mutants showing increased GTP binding are more readily recruited to the TGN, where they are activated by Rab29 binding to the ankyrin domain of LRRK2. GTP-bound LRRK2 bound by Rab29 shows increased kinase activity towards the S1292 residue of LRRK2 and Rab10, and is associated with increased phosphorylation of the N-terminal S910, S935, S955 and S973 residues. LRRK2 also phosphorylates Rab29, thereby inhibiting Rab29-mediated activation of LRRK2 kinase activity in a negative feedback loop.

Wauters et al. (2017) proposed a mechanism for the G-protein cycle in LRRK2 activation and localisation (Fig. 3F). In their model, GTP-bound LRRK2 is stabilised by 14-3-3 that binds phosphorylated N-terminal serine residues. Cytosolic GDP-bound Rab proteins stabilised by GDIs are activated by prenylation, and Rab GEFs mediate the exchange of GDP for GTP, leading to GTP-bound Rab proteins that become embedded in their target membrane. Membrane-embedded Rab proteins recruit LRRK2 by binding the N-terminal domains of LRRK2, leading to the activation of LRRK2 kinase

activity, triggering the phosphorylation of Rab proteins in their switch II domains as well as autophosphorylation of the LRRK2 S1292 residue. Rab phosphorylation hinders interaction with Rab GAPs, thereby slowing down Rab GTP hydrolysis. Meanwhile, or prior to activation of LRRK2 kinase activity, membrane-association of LRRK2 induces LRRK2 GTP hydrolysis, which causes dimerisation and activation of LRRK2.

A most recent model by Schmidt et al. (2021) highlights the function of the kinase domain in regulating LRRK2 activation. Schmidt at al. proposed that LRRK2 regulation by Rab GTPases is similar to BRAF activation by Ras GTPases. In their mechanism, the N-terminal domains of LRRK2 act as a "lid" for the kinase domain, keeping it in a closed conformation. When Rab proteins are activated and move to the membrane, they there recruit LRRK2 and bind to the N-terminal domains. Binding by Rab induces a conformational change that allows autophosphorylation of the S1292 residue. The kinase domain is freed, and can toggle between active and inactive states. In the active conformation, LRRK2 dimerises, stabilising the conformation in which the R-spine is assembled, allowing LRRK2 to phosphorylate different substrates. According to Schmidt et al. (2021), LRRK2 is kept stable in the cytosol because of the N-terminal "lid", further stabilised by phosphorylation-dependent 14-3-3 binding.

5.2 Self-proposed mechanism

Several themes of the above mechanisms overlap, while others differ. For instance, there seems to be an agreement on the regulation of Rab29 and Rab29-mediated activation of LRRK2 at membranes, while the G-protein cycle of LRRK2 and regulation of its oligomerisation are not reconciled. I propose a model in which LRRK2 is inactive, monomeric in the cytosol, stabilised by phosphorylation-dependent 14-3-3 binding as well as the N-terminal "lid" of LRRK2. GDP-bound Rab GTPases are stabilised in the cytosol by GDIs. Upon activation, Rab GTPases become prenylated, dissociate from their GDIs, and become embedded in their target membrane. GEF-mediated exchange of GDP to GTP activates the Rab, which then binds the ankyrin domain of LRRK2. A conformational change in LRRK2 partly releases the N-terminal "lid", allowing dephosphorylation of the 14-3-3 binding site coupled to its dissociation, as well as inducing autophosphorylation of the S1292 residue. The kinase domain is freed, inducing dimerisation of LRRK2. Dimerisation of LRRK2 induces GTP hydrolysis, relaying a conformational change in LRRK2 that further activates the kinase domain. The fully activated kinase domain phosphorylates both upstream and downstream Rab substrates as well as residues in the Roc domain of LRRK2 and stimulates phosphorylation of the N-terminal 14-3-3 binding site. Phosphorylation of upstream Rab GTPases alters their affinity towards effector proteins, resulting in GDP hydrolysis by Rabs, as well as dissociation of LRRK2 coupled to its monomerisation and inactivation. LRRK2 is again stabilised in the cytosol by its N-terminal "lid" and 14-3-3 binding.

One caveat in this model is that it does not account for the change in LRRK2 kinase activity observed in GTP-bound LRRK2 compared to GDP-bound LRRK2 (Biosa et al., 2012). One possibility is that both GTP-bound and GDP-bound version of LRRK2 follow the same mechanism of activation, except for GTP hydrolysis. GDP-bound LRRK2 can still partly activate kinase activity, while the GTP hydrolysis in GTP-bound LRRK2 might activate LRRK2 kinase activity to a greater extent. Similarly, as LRRK2 kinase activity seems to be regulated by Rab proteins, dimerisation, autophosphorylation, and GTPase activity, alterations of each of these elements might alter kinase activity, while a concerted effort may be required for full kinase activation.

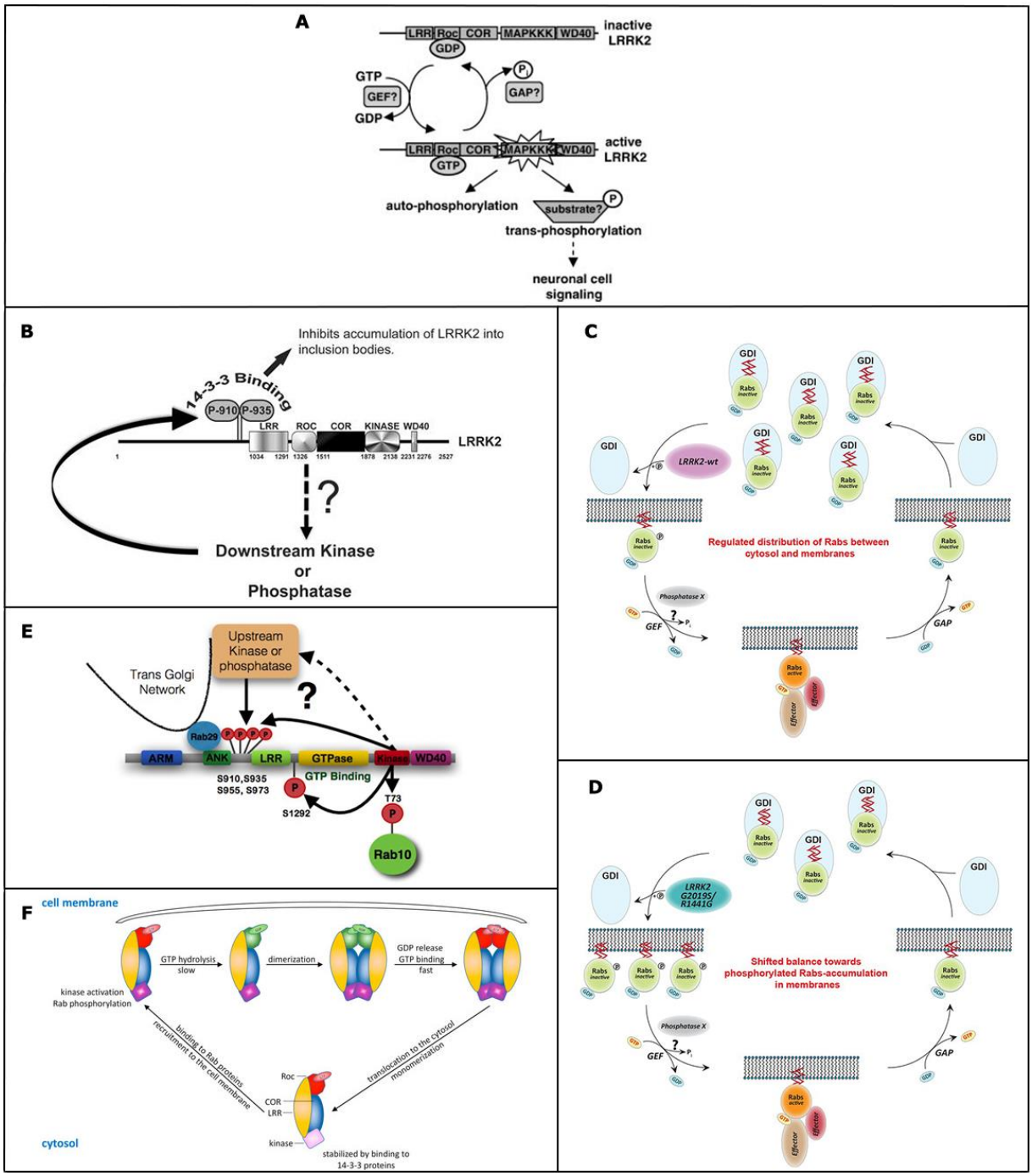

Figure 3: Proposed mechanisms found in literature. A) Mechanism proposed by Guo et al. (2007), in which LRRK2 cycles between a GDP-bound inactive state and GTP-bound active state, facilitated by GEFs and GAPs. B) Model by Dzamko et al. (2010), in which LRRK2 regulates downstream kinases or phosphatases that act on S910 and S935, thereby regulating the binding of 14-3-3. C) Model by Steger et al. (2017) in which inactive, GDP-bound Rab GTPase are bound in the cytosol by GDIs. LRRK2 mediates the dissociation of Rab GTPases from GDIs by phosphorylation and aids the insertion of GDP-bound Rab proteins in their specific target membranes. Membrane-bound Rabs are dephosphorylated, and GEFs facilitate the exchange of guanine nucleotides, thereby activating the now GTP-bound Rab proteins, which interact with effector proteins. Rab-specific GAPs induce GTP hydrolysis, followed by the removal of the Rab proteins from the membrane by GDIs. D) In their adapted model for mutant LRRK2, Steger et al. (2017) propose that hyperactive LRRK2 leads to reduced affinity of Rab GTPases to GDIs. As a result, the Rab GTPases become increasingly membrane-bound and active, leading to pathogenic phenotypes. E) Model by Purlyte et al. (2018) in which GTP-bound LRRK2 is recruited to the TGN. Rab29 binds to the ankyrin (ANK) domain of LRRK2, leading to phosphorylation of the S1292 residue of LRRK2, Rab10, and either direct or indirect phosphorylation of the N-terminal serine cluster of LRRK2. F) Model by Wauters et al. (2017) in which GTP-bound LRRK2 is stabilised by phosphorylation-dependent 14-3-3 binding. Membrane-embedded Rab proteins bind LRRK2, thereby recruiting LRRK2 to the membrane, where it is activated and phosphorylates Rab GTPases. GTP hydrolysis of LRRK2 induces a conformational change and leads to LRRK2 dimerisation. GDP is released, GTP is bound again and LRRK2 relocalises to the cytosol, where it forms monomers stabilised by 14-3-3 binding.

6. Conclusion

Though the cellular functions of the PD-associated LRRK2 are becoming clearer, its regulation and activation remain enigmatic. The presence of both a MAPKKK-like kinase domain and a Ras-like GTPase domain in LRRK2, which form well-characterised intermolecular interactions when present in individual proteins, insinuate an interplay between them in LRRK2 (Lavoie & Therrien, 2015). While the identification of Rab GTPases as LRRK2 substrates and the discovery that Rab29 functions as an upstream regulator of LRRK2 has helped shed light on some mechanistic features of LRRK2 activation, the nature of the G-protein cycle of LRRK2 remains unresolved (Purlyte et al., 2018). There is good evidence that LRRK2 forms active dimers, suggesting LRRK2 belongs to the GAD-type of G-proteins (Gasper et al., 2009). However, the identification of GEFs and GAPs interacting with LRRK2 suggests LRRK2 might behave as a classical G-protein instead (Nguyen & Moore, 2017). Several studies show that GTP binding may enhance LRRK2 activation and dictate its localisation, while data of LRRK2 homologs suggest LRRK2 forms an inactive monomer when bound to GTP, while the GDP-bound and nucleotide free versions of LRRK2 represent dimers (Blanca Ramírez et al., 2017b; Deyaert et al., 2017). The phosphorylation status of the N-terminal serine cluster consisting of S910, S935, S955 and S973, is frequently paralleled by 14-3-3 binding and stabilisation of LRRK2 in an inactive conformation in the cytosol, while PD-associated mutations that induces LRRK2 relocalisation to filamentous structures seem to be largely driven by the extent of GTP binding (Blanca Ramírez et al., 2017b; Dzamko et al., 2010). The differences between GDP and GTP binding on LRRK2 kinase activity are also not reconciled, some studies showing increased kinase activity for GTP-bound LRRK2 compared to its GDP-bound form, while others find no difference in kinase activity (Biosa et al., 2011; Taylor et al., 2011).

Though a lot of details surrounding LRRK2 activation and regulation remain to be resolved, several lines of evidence suggest an interplay between LRRK2 kinase and GTPase domains. i) The extent of GTP binding to the Roc domain of LRRK2 seems to form an important regulatory element in kinase activation (Biosa et al., 2011; Blanca Ramírez et al., 2017b). ii) The RocCOR tandem domain itself as well as guanine nucleotide binding seems to influence LRRK2 dimerisation, which is linked to kinase activation (Civiero et al., 2017; Gasper et al., 2009). iii) Autophosphorylation of Roc domain residues by the kinase domain seems to contribute to LRRK2 regulation (Liu et al., 2015). iv) GTP hydrolysis seems to influence kinase activity (Biosa et al., 2012; Nguyen et al., 2020).

Driven by the kinase-enhancing effect of the most prevalent LRRK2 G2019S mutant, current treatment of PD is mainly focused on inhibition of LRRK2 kinase activity. However, the highly complex regulatory mechanisms of LRRK2 activation described here illustrate that kinase inhibitors might influence more than just LRRK2 kinase activity. A deeper understanding of the interplay between LRRK2 kinase and GTPase domains will contribute to our understanding of LRRK2 regulation, and will therefore be invaluable for selective pharmaceutical intervention of PD.

7. References

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